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PRINCIPAL INVESTIGATOR: Lily Yang, M.D., Ph.D.

CONTRACTING ORGANIZATION: Emory University
Atlanta, Georgia 30322

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6. AUTHOR(S)
Lily Yang, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Emory University
Atlanta, Georgia 30322

E-Mail: lyang02@emory.edu

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13. ABSTRACT (Maximum 200 Words)

During the last funding year, our research focus has been on the design and synthesis of MBs targeting survivin, cyclin D1 and Her-2/Neu mRNAs and examination of the specificity of those MBs in human breast cancer cells. Our results showed that survivin and cyclin D1 MBs are able to produce strong fluorescence signals in breast cancer cells but not in normal cells. We also developed a procedure to detect both survivin and cyclin D1 gene expression simultaneously in single cancer cells, which increases the sensitivity and specificity of detection of cancer cells. Importantly, our study also demonstrated that the fluorescence signals produced from the hybridization of MBs with their specific mRNAs correlate very well with the level of gene expression in the cells, suggesting that MB-detection may have a broad use for detection of tumor marker genes since many of those marker genes are also present at a low level in normal cells and the differences between cancer and normal cells are just levels of gene expression. Based on the results of this study, we have submitted a manuscript entitled "Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies for Cancer Research" to Cancer Research.

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Breast tumor markers, Her-2/neu, cyclin D, surviving, molecular beacons, molecular imaging of cancer cells, early detection of breast cancer, identification of ductal carcinoma in situ, ductal lavage, fine needle aspiration, women at high risk of breast cancer

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Introduction:

The goal of this research project is to examine the feasibility of detection of breast cancer cells in fine needle aspiration and ductal lavage using the molecular beacon-imaging tumor marker gene expressing cells. The molecular beacon (MB) is a dual-labeled short hairpin oligonucleotide probe that produces a fluorescent signal only when hybridizing to specific nucleotide sequences[1]. We have previously developed this technology in our laboratory and have shown that the molecular beacon probe designed to target the expression of survivin gene, specifically survivin mRNA, is able to produce a strong fluorescence signal in human tumor cells but not in normal cells. To increase sensitivity and specificity of detecting cancer cells, we proposed to use the MBs to detect three tumor marker mRNAs that are present in over 80% of the early stage of breast cancer, ductal carcinoma *in situ*, including survivin, cyclin D1 and Her-2/Neu [2,3]. During the last funding year, we have been working on the design and analysis the specificity of the MBs for those three genes, establishment of experimental procedures for detecting the expression of multiple genes simultaneously in tumor cells and examination of correlation between the fluorescence signals produced from the MBs and the level of gene expression in human breast cancer cell lines. These *in vitro* works in human tumor cell lines established a foundation for us to carry out the human trial.

Body of the progress report

1. Design of the MBs and examination of specificity with their DNA targets and breast cancer cell lines.

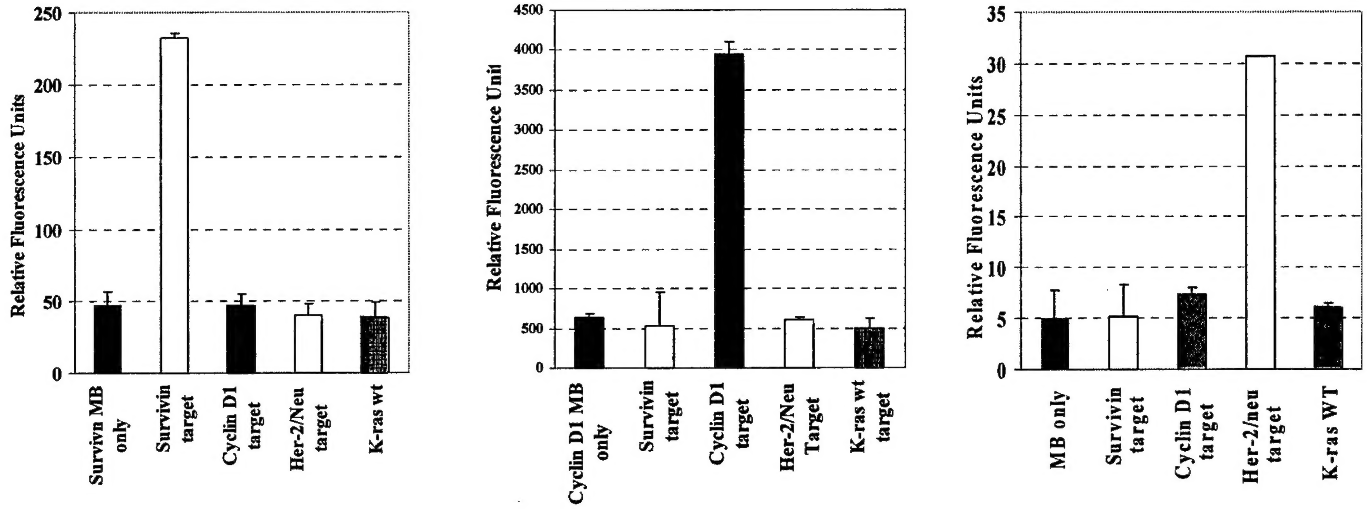
To detect the expression of survivin, cyclin D1 and Her-2/Neu genes, we examined the cDNA sequences of the genes and selected the regions of gene sequences that are the best for the MB design. The MBs for those three genes are shown in the Table-1. We also made an internal control MB targeting a GAPDH mRNA.

Table-1 Design of MBs targeting breast cancer marker genes

MBs	Sequences
Survivin MB	5'-FITC-TGGTCCTTGAGAAAGGGCGACCA-dabcyl-3'
Cyclin D1	5'-Texas-red-TGGAGTTGTCGGTGTAGACTCCA-dabcyl-3'
Her-2 Neu	5'-Alexa Fluor 350-CAGCTTCATGTCTGTGCCAGCTG-dabcyl-3'
GAPDH	5'-cy3-CGAGGTCCTTCCACGATACCACTCG-dabcy-3'

Next, we examine the specificity of those MBs hybridize to their DNA targets, our results showed that these MBs specifically hybridized to their target DNA sequences (Figure 1).

Figure 1. Specificity of the MBs for their DNA gene sequence detected in solution.



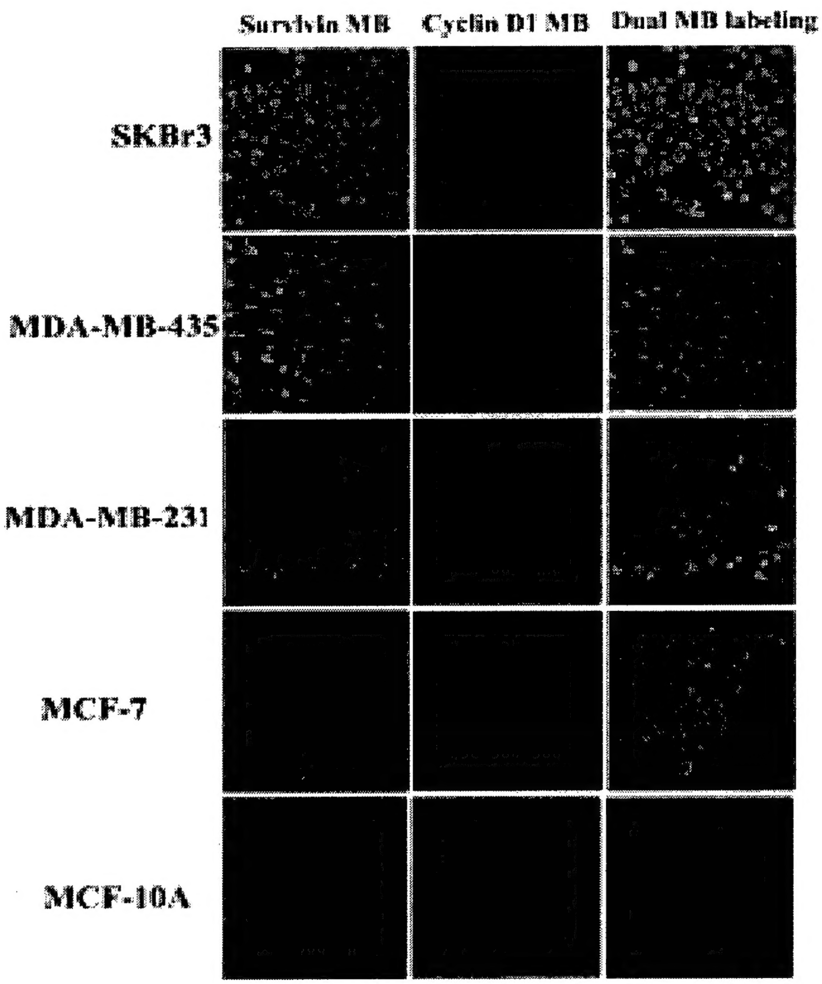
Survivin MB **Cyclin D1 MB** **Her-2/Neu MB**

Survivin, cyclin D1 or Her-2/Neu MB was mixed with various synthesized DNA targets. The fluorescence units were measured using a fluorescence microplate reader. Survivin or cyclin D 1 MB only bound and generated strong fluorescent signal when mixed with its specific DNA target. MBs have a high specificity in hybridizing to their gene targets.

We further examined the specificity of the MBs on human breast cancer cell lines and found survivin and cyclin D1 specifically produced the fluorescence signal in human breast cancer cell line but not in normal cells. Importantly, we examined the feasibility of simultaneously detection of the expression of both survivin and cyclin D1 in human breast cancer cells using the MBS with different fluorescence-dyes. We found that the MBs are capable of detecting the expression of two genes in single cells at the same time (Figure 2). However, delivery of the MBs into normal breast epithelial cells failed to produce fluorescence singles, suggesting those MBs are specific for tumor cells. Interestingly, we have demonstrated that the fluorescence intensities produced from the hybridization of the MBs with specific mRNAs correlate very well with the levels of the gene expression detected by real-time RT PCR and the protein levels detected by Western blot analysis (Figure 3). This finding is very significant for using MBs for detection of

human cancer cells since many the expression of tumor marker genes is not unique for tumor cells and the difference between normal and cancer cells is only the level of gene expression. The ability of quantitative detection of gene expression *in situ* should allow us to detect the tumor cells with a higher specificity.

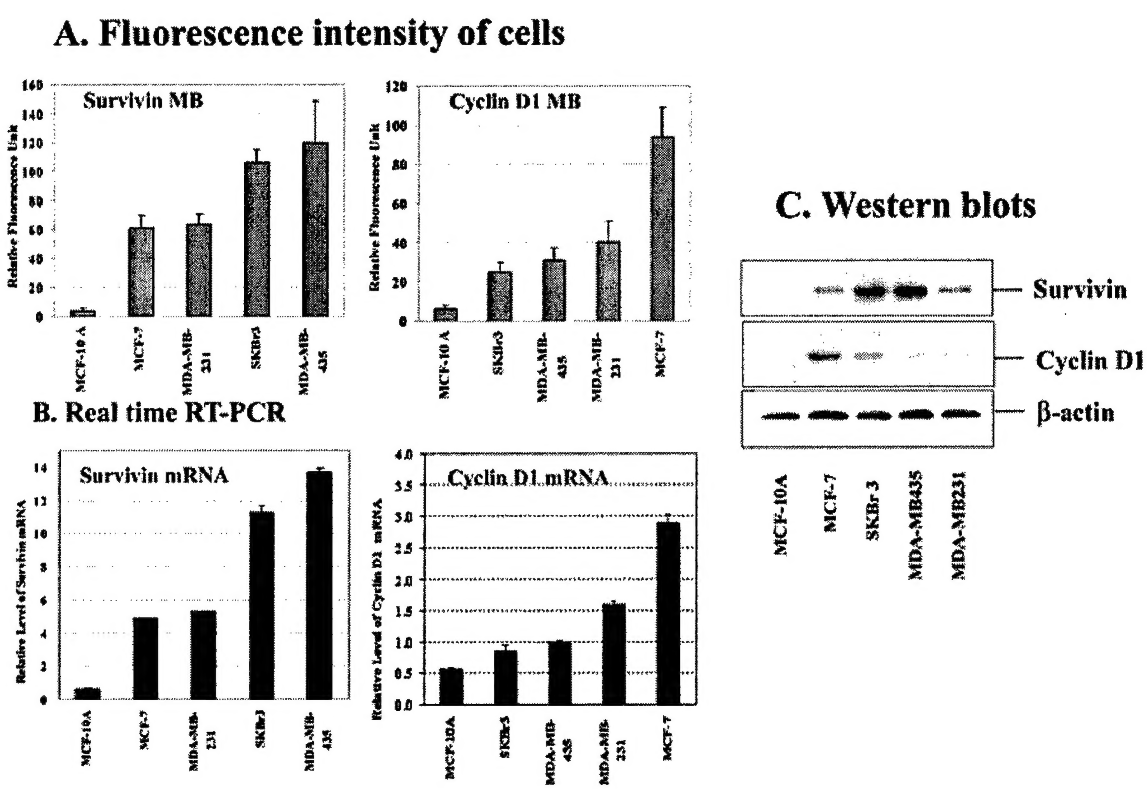
Figure 2. Detection of tumor cells expressing survivin and cyclin D1 genes simultaneously using the MBs.



Human breast cancer and normal cells were cultured in chamber slides and fixed with ice-cold acetone. A mixture of survivin and cyclin D 1 MBs was incubated with the fixed cells and then examined under a confocal microscope. Fluorescence images were taken as described in the Methods. The same exposure conditions were used for each color of the fluorescent dye. Survivin MB-FITC generated a strong green fluorescent signal in

most breast cancer cell lines but not in a normal mammary epithelial cell line (MCF-10A). Cyclin D 1 MB-Texas Red produced red fluorescent signals in breast cancer cell lines with the fluorescence intensities ranging from low to very strong. However, the fluorescent signal was very weak or negative in MCF-10 A cells after delivery of the cyclin D1 MB.

Figure 3. Quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells and correlation with results from real time RT-PCR and Western blots.



A. Fluorescence intensity was determined by measuring the mean fluorescence units from four randomly selected areas for each image taken under a confocal microscope. The mean fluorescence unit from four areas of each cell lines is shown in the figure. Similar results were observed in repeat experiments. **B.** Relative level of survivin or cyclin D1 mRNA was calculated from a ratio of the quantity of survivin or cyclin D1 PCR products and the quantity of β-actin PCR products. The real time RT-PCR results on the levels of survivin or cyclin D1 mRNA in different tumor and normal cell lines correlated very well with the fluorescence intensities detected in

the cell lines using MB detection (a and b). **C.** Examination of the levels of survivin protein in tumor and normal cell lines by Western blotting. The levels of survivin or cyclin D 1 protein correlated well with the levels of survivin or cyclin D1 mRNA detected *in situ* in fixed tumor cells using MB detection or with the real-time RT PCR results.

To determine the feasibility of detecting human breast cancer cells in clinical samples, we examined survivin-gene expressing cells in frozen tissue sections of human breast cancer tissues. We found that survivin MB, labeled with a cy3 fluorescence dye (red), is able to detect breast cancer cells in human tissues. We observed strong fluorescence signals in breast cancer tissues in DCIS, invasive and lymph node metastases but not in normal breast cancer tissues (Figure 4). Therefore, MBs can be used for specific detection of human breast cancer cells obtained from beast cancer tissues.

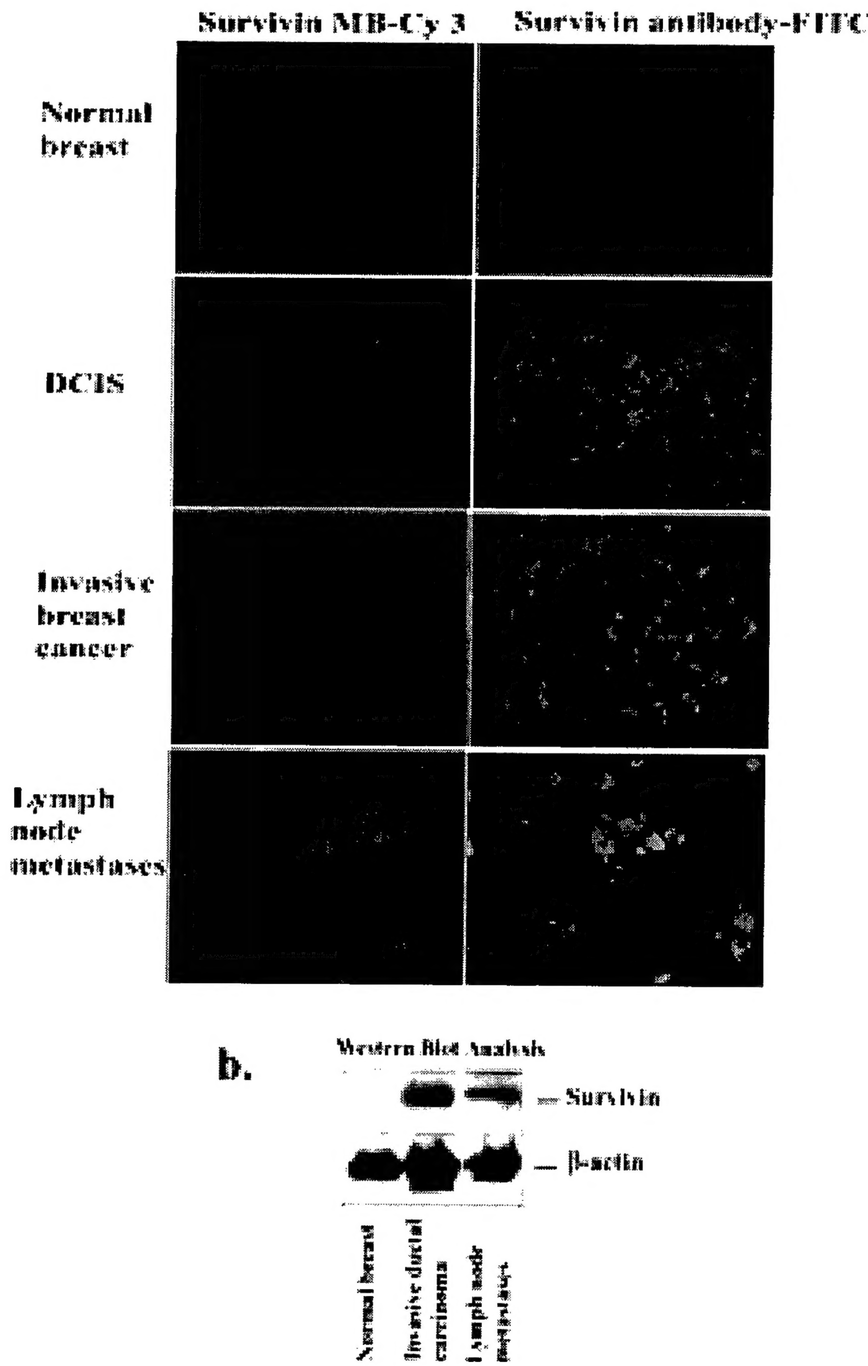
Figure 4. Detection of survivin gene expression on frozen tissue sections obtained from breast cancer patients.

a. Expression of survivin gene was detected in different stages of breast cancer tissues.

Frozen tissue sections were fixed with acetone and incubated with survivin MB-Cy3. The sections were counterstained with Hoechst 33342 (blue nuclei) and then examined under a fluorescence microscope. As shown, survivin-expressing cells (red) were found in all stages of breast cancer tissues including DCIS, invasive carcinoma and lymph node metastases, but not found in normal breast tissues. Different sections from the same tissues were also stained with a survivin antibody to confirm the presence of survivin positive cells (green). Yellow arrows indicate the detection of metastatic breast cancer cells in a lymph node by survivin MB or antibody.

b. Western blot analysis showed a high level of survivin protein (16.5 KDa) in primary breast cancer and lymph node with metastases but not in normal breast tissues.

Figure 4

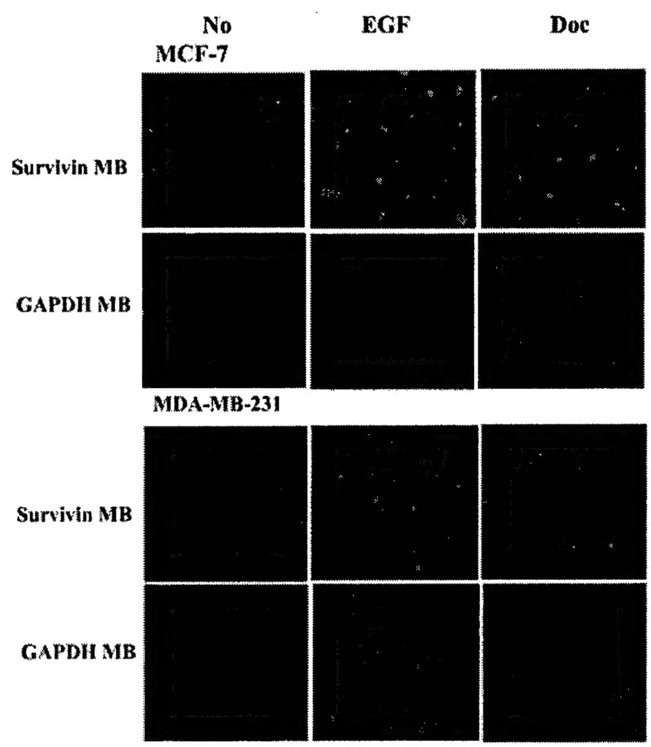


In addition to developing experimental procedures for fixed tumor cells, we also

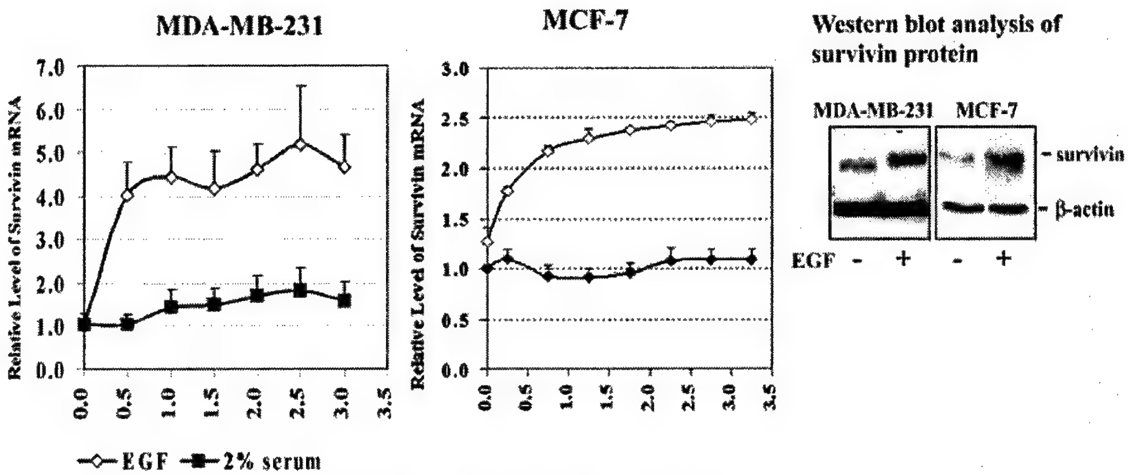
investigated the feasibility of using the MBs to detect the level of gene expression real time in living cells. We used two experimental models to stimulate the upregulation of survivin gene expression. It has been shown that chemotherapy drug docetaxel induces the expression of survivin gene expression in human breast cancer cells[4]. Our study also showed that epithelial growth factor (EGF)-treatment upregulates the survivin gene expression (unpublished results). We developed a procedure to transfect the MBs into viable cells at a high efficiency. We also found that fluorescence intensity produced in viable cells correlates with changes in the level of survivin gene expression in the cells (Figure 5 A. B and C). Development of this new method should allow us to examine the response of human breast cancer cells to biological and chemotherapy reagents real time *in vitro* using a simple procedure.

Figure 5. Detection of the levels of gene expression in living breast cancer cell lines.

A. Survivin MB-FITC produced green fluorescent signal in breast cancer cell lines after transfection into viable cells. The fluorescence staining for both survivin and GAPDH was located in cytoplasma as small clusters. Treatment of the cancer cells with EGF for 1 hr or docetaxel for 24 hrs increased the fluorescence intensity in survivin MB-transfected cells. On the other hand, the fluorescence intensity generated by GAPDH MB-Cy3, cotransfected with the survivin MB was relatively consistent in the cells.



B. Cells cultured in 96-well plates were transfected with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then added human EGF. The fluorescence intensity in each well was measured at different time points following treatment using a fluorescence microplate reader. EGF treatment significantly increased the level of survivin mRNA (Student's t-test for all time points, $P<0.0005$). Each point in the curve is a ratio of the mean fluorescence unit of survivin MB (FITC, Ex/Em 480/530) and mean fluorescence unit of GAPDH MB (Cy3, Ex/Em 530/590) from four repeat samples. Similar results were obtained in three independent studies. Analysis of the level of survivin protein by Western blotting further demonstrated that EGF increased survivin expression.

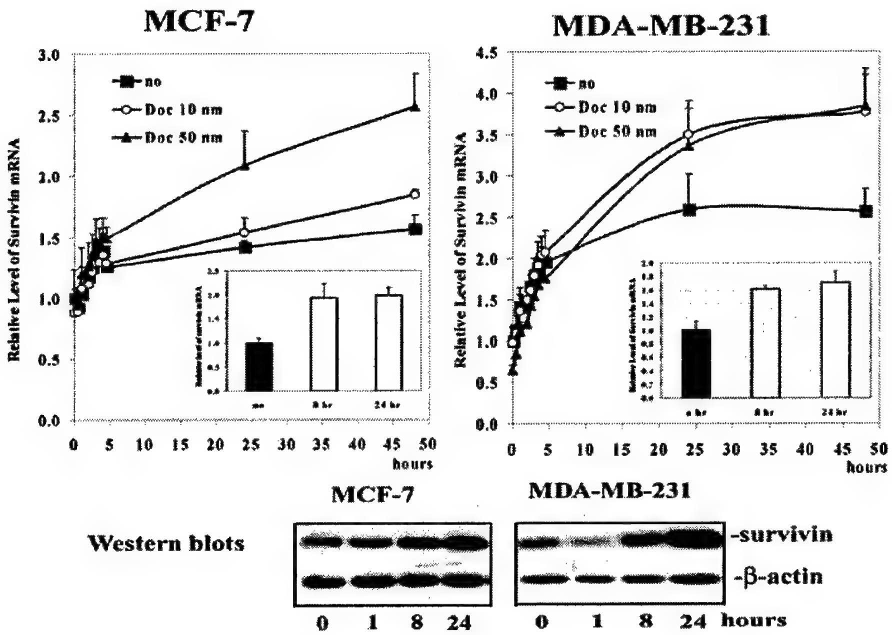


Student's t-test: MDA-MB-231 p= 0.0004; MCF-7, p=2.5x10E-8

C. Real-time detection of gene expression using survivin MB revealed upregulation of survivin gene expression in breast cancer cells after docetaxel treatment.

Cells cultured in 96 well plates were transduced with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then treated with 10 or 50 nM of docetaxel. Fluorescence units in each well were measured at different time points using a fluorescence microplate reader. Docetaxel treatment increased the level of survivin gene expression in both tumor cell lines and a significant increase in the level of survivin mRNA was seen 24 to 48 hrs following the treatment (Student's t-test, $P< 0.05$). Each point in the curve is a ratio of the mean fluorescence unit from survivin MB-FITC and GAPDH MB-Cy3. The mean fluorescence unit was calculated from four repeat samples and similar results were obtained from three independent studies.

The levels of survivin gene expression and survivin protein at different time points after treatment were also examined by real-time RT-PCR (insert in the figure) and Western blot analysis. The results from these experiments demonstrated upregulation of survivin gene expression by docetaxel.



Key Research Accomplishments:

1). We have design and synthesized the MBs targeting survivin, cyclin D and Her-2/neu mRNA. The specificity of these MBs in binding to targeting DNA sequences has been examined.

2). We have developed an approach to detect multiple tumor marker mRNAs simultaneously in single tumor cells.

3). We have demonstrated the specificity of survivin MB and cyclin D1 in detection of breast cancer cells in breast cancer cell lines and tissues.

4). We have shown a correlation between the fluorescence intensity produced from the MB probes and levels of gene expression in fixed as well as in viable tumor cells.

Reportable Outcomes

We have developed experimental procedures and conditions for carrying out human trial. At present, we have obtained an approval of the Human Investigation Committee of DOD for conducting the experiments in human breast cancer cell lines. We have modified our IRB protocol for the human study according to the requirement of DOD. We are ready to perform human study as soon as we obtain the final approval of our IRB protocol from the human Investigation Committee of DOD.

The goal of next funding year is to examine the feasibility of detection of the breast cancer cells using those MBs in clinical samples from patients and women at a high risk for developing breast cancer.

Conclusions

In summary, we have established and optimized our experimental conditions for detection of breast cancer cells using the MB-fluorescence imaging technology. We have ready for using this method for detection of cancer cells in clinical samples.

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Appendices

Manuscript submitted to Cancer Research, September, 2004

Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies and Implications for Cancer Research

XiangHong Peng, Zehong Cao, Jing-Tong Xia [ξ],

Grant Carlson, Melinda Lewis[#], William C. Wood and Lily Yang*

Departments of Surgery, Winship Cancer Institute, and [#] Pathology, Emory University School of Medicine, Atlanta, GA 30322, and [ξ] The First People's Hospital of Guang Zhou, P.R.China

*To whom correspondence should be addressed:

Dr. Lily Yang, Department of Surgery and Winship Cancer Institute

Emory University School of Medicine, 1365 C Clifton Road, N.E. Atlanta, GA 30322,

Phone: 404-778-4269, Fax: 404-778-5530, e-mail address: Lyang02@emory.edu

Running title: Molecular beacons for imaging cancer cells and detecting gene expression

Key words: Molecular imaging, Molecular beacon, Survivin, Cyclin D1, Breast cancer, cancer detection, fluorescence imaging and real-time gene expression

Abbreviation: MB: Molecular beacon, IAP: inhibitor of apoptosis protein, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, EGF: epidermal growth factor, Doc: docetaxel, RFU: Relative fluorescence unit, Dabcyl: 4-({4'-(dimethylamino)phenyl]azo)benzoic acid, DCIS: Ductal carcinoma *in situ*; MOI: Multiplicity of infection.

Abstract

Development of novel approaches for quantitative analysis of gene expression in intact tumor cells should provide sensitive and specific means for cancer detection and for studying the response of cancer cells to biological and therapeutic reagents. We developed procedures for detecting the level of gene expression in fixed as well as viable cancer cells using molecular beacon (MB) imaging technology. We found that simultaneous delivery of MBs targeting survivin and cyclin D1 mRNAs produced strong fluorescence in breast cancer but not in normal breast cells. Importantly, fluorescence intensity correlated well with the level of gene expression in the cells. We further demonstrate that MBs can detect changes of survivin gene expression in viable cancer cells following EGF stimulation, docetaxel treatment and overexpression of p53 gene. Thus, MB-imaging is a simple and specific method for detecting gene expression in cancer cells. It has great potential for cancer detection and drug development.

Introduction

Development of novel approaches for detecting cancer cells and determining the responses of the cells to therapeutic reagents holds great promise to increase the survival of cancer patients. It is well known that human cancer cells develop due to abnormalities in gene expression which provide growth advantages, metastatic potential and apoptosis resistance to the cells[1-3]. Methods for specific detection of abnormal gene expression in intact single cancer cells should provide new tools for identifying cancer cells in clinical samples, studying biological effects and evaluating the effects of therapeutic reagents on specific molecular targets in cancer cells.

In this study, we developed a molecular beacon (MB) fluorescence imaging approach to detect the levels of expression of multiple genes simultaneously in single cells. Molecular beacons (MBs) are stem-loop type oligonucleotide probes dual-labeled with a fluorophore and a quencher. In the absence of the target, the stem brings the fluorophore and quencher molecules together, which prevents the production of a fluorescent signal. When the MB hybrids to its specific target sequence, the stem is forced to break apart, which enables it to generate a fluorescent signal[4-6]. During the last several years, MB technology has been utilized in various applications to detect oligonucleotides in solution, including DNA mutation detection, real time quantification of PCR product and protein–DNA interaction[6-8]. Results of those studies have demonstrated that the MB detection is a reliable and specific approach for examining target oligonucleotides in solution.

The ability of MB probes to detect specific target molecules without washing away or separation of unbound probes also provides an opportunity to detect intracellular mRNA molecules in intact cells. The feasibility of detecting intracellular mRNA has been examined in

several laboratories [9-12]. It has been shown that MBs were able to visualize mRNA molecules in several human and animal cell lines after introducing into cells through microinjection or liposome delivery [9-11, 13]. Although previous studies suggested that detection of intracellular mRNA using MBs is a feasible approach, the question remains how to develop this novel technology into a simple procedure that can be used broadly in basic research and clinical laboratories. To address this issue, we developed procedures that enable us to detect gene expression in fixed as well as viable cells. We designed MBs targeting survivin and cyclin D1 mRNAs, which are highly expressed in breast cancer cells[14]. Survivin is a member of the inhibitor of apoptosis (IAP) protein family that plays a crucial role in the apoptosis resistance of tumor cells[15]. Increasing evidence indicates that survivin is also a promising tumor marker since it is normally expressed during fetal development but is not expressed in most normal adult tissues[16]. However, high levels of survivin are detected in many human cancer types including 71% of breast cancers [17, 18]. The role of survivin in blocking the apoptotic response and fostering resistance to chemotherapy drugs and radiotherapy by cancer cells has been well elucidated[19, 20]. Also, cyclin D 1, an important regulator of cell cycle, is overexpressed in 50-80% of breast cancer tissues whereas it is low or absent in normal breast tissues[14].

In this study, we examined the feasibility of detecting expression of survivin and cyclin D1 genes in human breast cancer cells using the MB-imaging technology. We found that the levels of fluorescence intensity detected in those tumor cells were correlated very well with the levels of survivin and cyclin D1 gene expression as detected by Real Time RT-PCR or Western blot analysis.

Results

Survivin and cyclin D1 MBs specifically bind to DNA targets

The designs for survivin and cyclin D1 MBs are shown in Fig. 1. a. We demonstrated that survivin or cyclin D1 MB specifically bound to its DNA target and generated strong fluorescent signals. Survivin MB produced a 5 to 6 fold higher fluorescent signal when mixed with survivin target compared with other DNA targets such as cyclin D1, Her-2/neu and K-ras (Fig. 1 b). Similarly, 7 to 8 fold higher fluorescent signals were detected in the mixture of cyclin D1 MB and cyclin D1 target but not in other groups (Fig. 1 b).

Detection of human breast cancer cells using MBs targeting tumor marker mRNAs.

We examined whether MBs targeting different tumor marker mRNAs can be labeled with different fluorophores and expression of the tumor marker genes can be determined simultaneously in single cells. Our results demonstrated that a combination of survivin and cyclin D1 MBs detected the expression of both survivin and cyclin D1 genes simultaneously and generate fluorescent signals corresponding to either survivin (green) or cyclin D1 (red) mRNA in the cancer cells (Fig. 2 a). Importantly, the fluorescent signal was very low for both survivin and cyclin D1 MBs in a normal immortalized human mammary epithelial cell line (MCF-10A), indicating that survivin and/or cyclin D1 MBs can be used as fluorescence probes for the detection of breast cancer cells (Fig. 2. a). The results of examination of fluorescence intensity and the level of survivin or cyclin D1 gene expression in tumor and normal cell lines further showed that the fluorescent signals detected by the MBs correlated very well with the levels of survivin or cyclin D1 gene expression, both in mRNA and protein levels (Fig. 2 b, c and d). For example, MDA-MB-435 and SKBr-3 expressed very high levels of survivin as detected by Real Time RT-PCR assay and Western blot analysis, and the strongest fluorescent signal was detected

in these cell lines. Conversely, these cell lines expressed low levels of cyclin D1 gene and showed a weak red fluorescence staining (Fig. 2 a, b, c and d). Another breast cancer cell line, MCF-7, expressed a moderate level of survivin gene but had a very high level of cyclin D1 gene expression (Fig. 2 a, b, c and d). Delivery of survivin and cyclin D1 MBs into this cell line produced a strong red fluorescent signal (cyclin D1) and an intermediate level of green fluorescent signal (survivin) (Fig. 2 a, b, c and d). Our results demonstrate that a combination of MB technology with fluorescence imaging is a novel approach to simultaneously detect the levels of multiple gene expressions in intact single cells.

MBs detect cancer cells on frozen sections of breast cancer tissues.

At present, pathological diagnosis of breast cancer in patient samples such as fine needle aspiration, core biopsy and surgically resected tissues mainly depends on morphological classification [21]. Although antibodies against various tumor markers such as Her-2/Neu and EGF receptor have been used to identify breast cancer cells, only 30 to 50 % of breast cancers express these tumor markers [22, 23]. However, it has been shown that over 70% of breast cancer tissues express a high level of survivin [17, 18]. We have developed a simple and fast procedure that allows us to detect survivin gene expression *in situ* on frozen tissue sections. We showed that survivin MB-Cy3 was able to produce strong red fluorescent signals in breast cancer cells (Fig. 3 a). Using this method, we found that survivin gene expression is an early event in the tumorigenesis of breast cancer, and is present in the ductal carcinoma *in situ* (DCIS) stage. A high level of survivin gene expression was also consistently detected in most breast cancer tissues of invasive ductal carcinoma and lymph node metastases but not in normal breast tissues (Fig. 3 a). In addition, we found that survivin gene-expressing cells in breast cancer tissues included cancer cells as well as cells in the vascular structures (Fig. 3 c). When the same section was double-

labeled with an antibody specific for a CD 31 human endothelial cell marker[24], those survivin-expressing cells were shown to be endothelial cells (Fig. 3 c). Establishment of this MB detection method at the level of gene expression *in situ* should provide pathologists with a revolutionary tool to identify cancer cells in clinical samples.

Monitoring the level of real-time gene expression using survivin MB.

Development of a sensitive method for determining the change of expression of specific genes will facilitate studies of cancer biology and the development of new cancer drugs, especially for therapeutic reagents targeting specific molecules in cancer cells. Unlike the traditional linear probes where unbound probes also generate fluorescent signals, MBs should only produce fluorescent signals when hybridized specifically to their target mRNAs, which makes them ideal probes for detecting the level of mRNAs in viable cells.

We used three model systems to determine whether survivin MB was able to detect changes of survivin gene expression in viable cells, including EGF or docetaxel induced upregulation, and tumor suppressor gene p53-induced downregulation of survivin gene expression [25, 26]. Human breast cancer cells were transfected with a mixture of survivin and GAPDH MBs and observed under a fluorescence microscope after treated with EGF for 1 hr or docetaxel for 24 hrs. Our results showed that treatment of the cells with EGF or docetaxel increased the level of survivin gene expression. Under a fluorescence microscope, the green fluorescence intensity (survivin MB-FITC) was stronger in the cells treated with either EGF or docetaxel compared to untreated control while the fluorescent signal for GAPDH MB (Cy3, red) was relatively consistent (Fig. 4 a). We further used FACScan analysis to determine the mean fluorescence intensity of all cell populations. Consistent with our observations with the fluorescence microscopy, we detected higher levels of fluorescent signal in EGF-treated cells

compared to the untreated group in both MCF-7 and MDA-MB-231 cells (Fig. 4 b). The relative level of survivin mRNA could be quantified from the FACScan data using the fluorescence unit of GAPDH gene as an internal control. We found that EGF treatment induced about 1.5 fold increases in the level of survivin gene expression in breast cancer cells.

In addition to the detection of levels of up-regulated genes, we examined the feasibility of quantifying the relative level of gene expression that was down-regulated in the cells. It has been shown that over-expression of p53 gene decreases the expression of survivin gene[26]. We transduced the tumor cells with Ad p53 vector or control vector AdCMV for 24 hrs and then delivered survivin or GAPDH MBs into the transduced cells. Using FACScan analysis, we found that the relative fluorescence was decreased about 2 fold in Ad p53 vector- transduced cells compared to the untreated or empty AdCMV vector control group (Fig. 4 c). The ability of MBs to detect the level of gene expression decrease in the cells suggests that the fluorescent signals detected intracellularly after MB transfection were not from non-specific degradation of the MBs, since the same amount of survivin and GAPDH MBs were delivered into Adp53 and control vector-transduced cells. Our results from Real time RT-PCR further confirmed that EGF increased the transcription of survivin gene and overexpression of the p53 gene decreased the level of survivin mRNA (Fig. 4 d).

Although detection of the level of gene expression by FACScan could accurately measure the fluorescence intensity in individual cells as well as in cell populations, the procedure for FACScan is time-consuming and does not easily detect changes of gene expression real-time in the same cell population. To develop a high throughput method for monitoring the changes of gene expression real-time in viable cells, we examined the feasibility of detecting levels of gene expression in cells cultured in 96-well plates using the MB-transfection approach. Breast cancer

cells were plated in 96-well plates and transfected with a mixture of survivin and GAPDH MBs for 3 hrs. After adding EGF or docetaxel, the plates were immediately placed in the fluorescence microplate reader and the fluorescence units in each well were measured at different time points. We found that EGF-induced up-regulation of survivin gene expression occurred as early as 15 minutes following the treatment and lasted for over 3 hrs (Fig. 5 a). There were 2.3 (MCF-7) to 2.8 (MDA-MB-231) fold increases in the relative levels of survivin mRNA after EGF treatment. We also examined the level of survivin protein using Western blot analysis and further confirmed that the level of survivin protein increased following EGF treatment.

For real-time detection of the level of gene expression in viable cells, it is important to determine how long the MB probes will stay in the cells and still be able to produce fluorescent signals that reflect the relative level of the gene expression. It has been shown that chemotherapy drug docetaxel increases in the level of survivin gene expression as early as 4 hours following the docetaxel treatment [25]. We examined the level of survivin gene expression real time in survivin and GAPDH MB-transfected cells following docetaxel treatment from 0 to 48 hrs. We found that compared to untreated cells, the level of survivin mRNA was increased at 5 hrs and reached a higher level 24 and 48 hrs after treatment (Fig. 5 b). The relative level of survivin mRNA is about 1.5 fold higher in docetaxel-treated cells than control cells and the difference detected 48 hrs after docetaxel treatment is statistically significant (Student's t-test, $P< 0.05$ for both MCF-7 and MDA-MB-231 cell lines). We also found a similar level of increase in the level of survivin mRNA detected by Real time RT PCR compared to survivin MB detection and the level of increase in survivin protein after docetaxel treatment (Fig. 5 b; insert is Real-time RT-PCR result.).

One of the important issues to be addressed in developing an oligo-based approach for detecting gene expression in viable cells is whether the binding of the MB probes to their target RNA leads to degradation of the mRNA by RNAase H, which may affect the level of target mRNA [27]. To answer this question, we transfected breast cancer cells with either survivin MB or control GAPDH MB for 24 hrs, and then examined the level of survivin protein by Western blot analysis. We found that compared to cells transfected with a non-specific GAPDH MB, presence of the survivin MB in the cells did not have a significant effect on the level of survivin protein (Fig. 5 c). At present, the mechanisms for retaining the level of survivin protein in survivin MB-transfected cells are still to be determined.

Discussion

We have developed a novel MB-based molecular imaging approach that allows identification of tumor cells expressing specific marker genes. We demonstrated that survivin and cyclin D1 MBs can be used to detect breast cancer cells and to determine the relative level of gene expression in viable cancer cells.

Human cancers contain heterogeneous cell populations with various genetic changes [28]. Simultaneous detection of over-expression of several tumor marker genes, especially when a single cell expresses more than one marker gene, may have a high predicative value for identifying cancer cells, and therefore increase the sensitivity and specificity of cancer detection. Since MB is highly specific in detecting target mRNAs, and MBs targeting various genes can be labeled with different fluorescent-dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. Using MBs targeting survivin and cyclin D1 mRNAs, we demonstrated that delivery of a mixture of survivin and cyclin D1 MBs into fixed cells produced fluorescent signals in breast cancer cells but not in

normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative since the signals were amplified by either the presence of multiple fluorescent-dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Since each MB has only one fluorophore and unbound MBs do not fluoresce, the fluorescence intensity generated by hybridization of the MB with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells.

The quantitative measurement of mRNA levels by MBs is very important for the future use of this technology for cancer cell detection since many tumor marker genes are not unique to cancer cells and the difference between normal and cancer cells can be only the level of gene expression. Although we used two MBs to detect the expression of tumor marker genes, a proof of principle from this study will lead to the use of more MBs with multiple dye molecules to analyze the expression of several tumor genes. Additionally, since only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared to conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Current methods for the identification and classification of cancer cells from clinical samples rely on examining the morphology of the cells or immunostaining with antibodies for tumor-related protein markers. Although the *in situ* hybridization using labeled linear probes has been used to detect gene expression on tissue sections, it is very time-consuming and usually

accompanied by a high background since unbound probes also produce fluorescent signals. In our study, we found that MBs could be used to detect the expression of genes on frozen tissue sections. The procedure is very simple and results can be examined within 30 to 60 minutes without the extensive staining and washing steps as required by the *in situ* hybridization protocol. Demonstration of the feasibility of combining the MB and immunofluorescence approaches to examine the levels of gene expression and protein *in situ* in the same cell population makes its potential application in pathological diagnosis of human cancers more appealing.

At present, MB technology has been mainly used in various applications *in vitro*, which were performed in solutions with defined MB-target conditions. For example, MBs targeting a breast cancer gene BRCA 1 were coated on a miniature biochip to detect the presence of BRCA 1 in solution[29]. Although previous studies showed the feasibility of detecting mRNAs and monitoring the transportation of RNAs in cells, the procedure for delivery of the MBs through microinjection into individual cells or by liposome delivery has made it difficult to apply this technology into broad research areas or into a routine clinical procedure [9-12]. We developed this MB-based and high throughput procedure for the detection of gene expression in viable cells. We demonstrated that transfecting survivin MB into cells produces a strong fluorescent signal in survivin-expressing tumor cells and the level of survivin gene expression can be monitored real-time in cells either by FACScan or by using a fluorescence microplate reader. Using these methods, we detected an increase in the level of survivin gene expression following EGF and docetaxel treatment. Although we used GAPDH MB as an internal control for our experiments, simultaneous detection of survivin and GAPDH gene expression real time in viable cells

indicates that it is also feasible to monitor the levels of expression of several genes in the same cell population using MBs labeled with different fluorophores.

One concern in the delivery of unmodified MBs to viable cells is that the MBs may be digested by nucleases in the cells or non-specific interaction between MBs and cellular proteins may open up the stem of the MBs, resulting in non-specific fluorescence. However, our results showed that the fluorescence intensity detected either by FACScan or microplate reader correlated well with the level of survivin mRNA in the tumor cells. Since a similar level of the MBs in a mixture of survivin and GAPDH MBs was delivered into the tumor cells, it seemed that increases in the fluorescence intensity in EGF and docetaxel treated cells or a decrease in p53 expressing cells were not due to non-specific degradation of the MBs.

In this study, we demonstrated that MB imaging of tumor cells is a simple and specific approach for the detection of breast cancer cells. This study is the first to apply state-of–the art MB-based methodology for cancer cell detection and for real time monitoring the level of expression of tumor marker genes in viable cells. Based on this study, high throughput assays for measuring the expression of multiple genes critical for drug response can be developed for screening cancer drugs that target specific molecules or pathways in cancer cells. To increase the specificity of MB detection, the MBs can be further modified to make them resistant to nuclease or RNAase H, such as by using 2'O-Methyl MB probes, or detecting fluorescence resonance energy transfer from two MBs that hybridize to nearby mRNA sequences [12].

Methods

Human tumor and normal cell lines.

Breast cancer cell lines SKBr-3, MDA-MB-231 and MCF-7, and normal immortalized human mammary epithelial cell line MCF-10A were obtained from the American Type Culture

Collection (ATCC, Manassas, Virginia). Another breast cancer cell line, MDA-MB-435, was provided by Dr. Zhen Fan (M.D. Anderson Cancer Center, Houston, Texas). Human breast cancer cell lines were maintained in DMEM/F-12 medium (50:50, Mediatech, Herndon, Virginia). All the above media were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Mediatech). MCF-10A cells were cultured in DMEM/F12 medium supplemented with 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisome, 100 ng/ml of cholera toxin, 10 μg/ml insulin, 2 mM L-glutamine and 5% FBS.

Human normal and breast cancer tissues.

Frozen human breast cancer and normal tissues were obtained according to an approved IRB protocol at Emory University from breast cancer patients during surgery to remove the tumors. The pathology of breast tissues was confirmed by pathologists at Emory University. Tissues were frozen immediately in liquid nitrogen and stored in a -80^{0}C freezer for further study.

Design and synthesis of MBs

The sequences of MBs targeting survivin or cyclin D1 mRNAs, selected from the GenBank, were unique for each gene. All survivin MBs contain the sequence regions with non-homology with other members of the inhibitor of apoptosis proteins. These include: 1) survivin MB-FITC (33 to 50 nt of the gene): 5'-FITC-TGGTCCTTGAGAAAGGGC<u>GACCA</u>-Dabcyl-3'; 2) Survivin MB-Cy3 (27 to 43 nt of the gene): 5'-Cy3-<u>C</u>TGAGAAAGGGCTGCCAG<u>TCTCAG</u>-Dabcyl-3'; and 3) Cyclin D1 MB-Texas red (375 to 393 nt of the gene): 5'-Texas-red-TGGAGTTGTCGGTGTAGA<u>CTCCA</u>-Dabcyl-3'. The design of the survivin and cyclin D1 MBs and illustration of the mechanism of binding MBs to specific oligonucleotide targets are shown in Fig. 1 a. Control MBs for targeting human glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) gene at 503 to 521 nt, GAPDH MB-Cy 3 or GAPDH MB-6-FAM, were also synthesized as the following: 5'-Cy3 or 6-FAM-CGAGTCCTTCCACGATACCACTCG-Dabcyl-3'. For each MB design, the stem region has 5 to 6 nucleotides. Sequences of one arm of the stem and the loop region of the MBs were complementary to a segment of the target genes. The underlined bases were those added to form a stem with an optimal Tm condition. All MBs were synthesized in MWG-Biotech Inc. (High Point, North Carolina).

Determination of specificity of the MBs binding to their oligonucleotide targets in solution.

The oligonucleotide targets for each MB were synthesized at Sigma Genosys, Woodlands, Texas. These include: 1) survivin target (13 to 72 nt): 5'-CCTGCCTGGCAGC CCTTTCTCAAGGACCACCGCATCTCTACATTCAAGAAC-3'; 2) cyclin D 1 target (364 to 399 nt): 5'-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC-3'; 3) Her-2/Neu gene target (72 to 108 nt): 5'-AGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCCT-3'; and 4) K-ras gene (22 to 78 nt): 5'-GTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGA CGATACAGCTAATT CAG-3'. Her-2/Neu and K-ras gene targets were used as non-specific target controls.

Specific binding of survivin or cyclin D1 MB to various DNA targets was determined by mixing 200 nM of the MBs with 1 μM of oligonucleotide targets in 100μl of Opti-MEM medium (Invitrogen, Carlsbad, California) in 96-well plates. After incubating at 37^0C for 60 minutes, fluorescence intensity in the mixture of 96 well was measured by a fluorescence microplate reader (Bioteck FL600 Fluorometer, Winooski, Vermont).

Real time RT-PCR

Total RNAs were isolated using a RNA Bee kit (Tel-test, Friendswood, TX). 2 μg of RNA samples were amplified with an Omniscript RT kit using an oligo dT primer (QIAGEN

Inc, Valencia, California) to generate 20 μl of cDNAs. 1 to 2 μl of cDNA was then quantified by real time PCR with primer pairs for survivin, cyclin D1, β-actin, or GAPDH using SYBR Green PCR Master mix. Real-time PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, California). The primer pairs for detecting the expression of survivin gene were survivin forward, 5'-TCCACTGCCCCACTGAGAAC-3' and survivin reverse, 5'-TGGCTCCCAGCCTTCCA -3', which amplify a 76 nt PCR product located from 130 to 206 nt of survivin mRNA. PCR primers for cyclin D1 (from 364 to 453 nt) were forward 5'-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC-3' and reverse 5'-GGTTCCACTTGAGCTTGTTCACAA-3'. Amplification of β-actin or GAPDH gene was used as an internal control for Real-time RT-PCR. The primer pair for β-actin gene were; β-actin forward, 5'-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3' and β-actin reverse, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3', which generate a 219 nt PCR product from 870 to 1089 nt of the β-actin mRNA sequence. Primer pairs for GAPDH (from 10 to 953 nt were: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCC ACCAC-3'. The quantity of PCR products from amplification of the survivin gene was standardized with the quantity of β-actin or GAPDH products for each sample to obtain a relative level of gene expression.

Western blot analyses

Cells were lysed in 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 10 mM $Na_2P_2O_7$ 10 H_20, 50 mM NaF, 1 mM $NaVO_4$, 1% Triton X-100 and protease inhibitor cocktail tablets (Complete mini, Roche Molecular Biochemical, Indianapolis, Indiana). 50 μg of protein was resolved on 12-15% polyacrylamide SDS gels and then transferred to PVDF membranes (Bio-Rad laboratories, Richmond, California). The membranes were blocked with 5% of non-fat milk

in Tris-buffered saline for 1 hour and then incubated for 2 hours with goat anti-human survivin (1: 600) (Santa Cruz Biotechnology, Santa Cruz, California) or mouse monoclonal anti-β-actin antibody (1:2000) (Sigma Chemical Co, St. Louis, Missouri). After three washings, the membranes were incubated with horseradish-peroxidase conjugated with anti-goat or mouse secondary antibody (Santa Cruz Biotechnology) for 1 hour. The levels of specific proteins in each lysate were detected by Enhanced Chemiluminescence using ECL plus (Amersham International, Buckingham, United kingdom) followed by autoradiography.

Detection of gene expression in fixed cells.

Human breast cancer cell lines and a normal immortalized mammary epithelial cell line, MCF-10A, were plated on chamber slides for 24 hrs and then fixed with ice-cold acetone for 5-10 minutes. After air drying, the slides were stained immediately or stored in a -80^{0}C freezer until use. A mixture of 200 nM of survivin MB-FITC and cyclin D1 MB-Texas red in Opti-MEM medium was incubated with the slides at 37^{0}C for 60 minutes. The slides were then examined using a confocal microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc., Thornwood, New York).

For detecting survivin gene expression on tissue sections, 5 μ frozen sections of breast normal and cancer tissues fixed with ice-cold acetone were incubated with 200 nM survivin MB-Cy3 for 60 minutes and then counter-stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugene, Oregon). The tissue slides were observed under a Nikon fluorescence microscope (Nikon Eclipse E800, Nikon Instruments Inc. Melville, New York). Fluorescence images were taken using an Optronics Magnafire digital imaging system (Meyer Instruments, Houston, Texas). For each experiment, we used the same exposure time to take all images for each color.

Therefore, fluorescence intensity for each image was consistent with the actual image observed under the microscope.

Immunofluorescence staining.

Acetone-fixed frozen sections were incubated with a polyclonal goat anti-human survivin antibody for 60 minutes (Santa Cruz Biotechnology). After washing with PBS, the slides were incubated with FITC-conjugated donkey anti-goat antibody for 30 minutes and then examined under a Nikon fluorescence microscope. For double labeling survivin MB and human endothelial cell marker CD31, frozen sections of an invasive breast cancer tissue were fixed and incubated with survivin MB-Cy3 and then with an anti-CD31 antibody followed by a FITC-conjugated secondary anti-mouse antibody. One tissue section was double-labeled with goat anti-human survivin and mouse anti-human CD31 antibodies. The secondary antibodies used were FITC-labeled donkey anti-goat antibody, or biotinylated-horse anti-mouse antibody followed by Texas-red avidin.

Quantification of the level of gene expression in viable cells using MBs

FACScan analysis: Cells cultured in 2% FBS medium or transduced with an adenoviral vector expressing a wild type p53 gene (Ad p53, Qbiogene, Carlsbad, California) or control adenoviral vector without gene (Ad CMV) at a multiplicity of infection (MOI) of 50 (PFU) for 24 hrs were collected by EDTA-trypsin digestion and divided into two groups. One group of the cells was transfected in suspension with 400 nM of survivin MB-FITC and the other was transfected with 400 nM of GAPDH MB-6-FAM using Lipofectamine 2000 in Opti-MEM medium (Invitrogen). GAPDH MB Cy3 was not used for this experiment since FACscan could not detect Cy3 fluorescent signal. Three hours following transfection, 100 ng of human recombinant EGF (Invitrogen) was added to EGF-treated group for 1 hr. After a brief wash with PBS, fluorescence

intensity of the cells from all groups was examined using FACScan analysis and the results were analyzed using Cell-Quest software (FACScan, Becton Dickinson, Mansfield, Massachusetts).

Fluorescence microplate reader: Cells were plated in 96-well tissue culture plates at 80% confluence for 24 hrs. All groups except the no-MB control group were then transfected with a mixture of 400 nM of survivin MB-FITC and internal control GAPDH MB-Cy 3 in Opti-MEM medium using Lipofectamine 2000. 3 hrs following transfection, basal levels of the fluorescence unit in the wells were measured using a fluorescence microplate reader (Bioteck FL600 Fluorometer). 100 ng of EGF was then added to the wells in the EGF–treated group and 10 to 50 nM of docetaxel (Aventis Pharma, Bridgewater, New Jersey) were added to docetaxel-treated groups. The culture plates were immediately placed in the microplate reader and fluorescence units in each well were measured every 30 min for 3 hrs. For docetaxel-treated groups, the plates were also measured at 24 and 48 hrs.

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Figure Legends

Figure 1. Schematic illustration of MB design and examination of specific binding of the MBs to their oligonucleotide targets.

a. Survivin and cyclin D1 MBs.

Both survivin and cyclin D1 MBs have 23 nt with 5'-stem and loop sequences complementary to survivin or cyclin D1 gene. The stem length for survivin MB is 5 nt with the 5'-end labeled with FITC and the 3'-end labeled with a quencher, Dabcyl. Cyclin D1 MB has a stem containing 6 nt with the 5'-end labeled with Texas-red and the 3'-end with Dabcyl. Survivin and cyclin D1 MB only generate fluorescent signals when hybridized to their specific DNA target.

b. Examination of specificity of the MBs *in vitro.*

Survivin or cyclin D1 MB was mixed with various synthesized DNA targets. The fluorescence units were measured using a fluorescence microplate reader. Survivin or cyclin D 1 MB only bound and generated strong fluorescent signal when mixed with its specific DNA target. MBs have a high specificity in hybridizing to their gene targets.

Figure 2. Simultaneous detection of the levels of survivin and cyclin D1 mRNAs in breast cancer cells.

a. Dual MB-imaging of breast cancer cells.

A mixture of survivin and cyclin D 1 MBs was incubated with the fixed cells and then examined under a confocal microscope. Fluorescence images were taken as described in the Methods.

b. Quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells.

Fluorescence intensity was determined by measuring the mean fluorescence units from four randomly selected areas for each image taken under a confocal microscope. The mean fluorescence unit from four areas of each cell line is shown in the figure. Similar results were observed in repeat experiments.

c. Detection of the levels of survivin and cyclin D1 mRNA by Real Time RT-PCR.

Relative level of survivin or cyclin D1 mRNA was calculated from a ratio of the quantity of survivin or cyclin D1 PCR products and the quantity of β-actin PCR products. Real Time RT-PCR results on the levels of survivin or cyclin D1 mRNA in different tumor and normal cell lines correlated very well with the fluorescence intensities detected in the cell lines using MB detection (a and b).

d. Examination of the levels of survivin protein in tumor and normal cell lines by Western blotting.

The levels of survivin or cyclin D 1 protein correlated well with the levels of survivin or cyclin D1 mRNA detected *in situ* in fixed tumor cells using MB detection or with the Real-Time RT PCR results.

Figure 3. Detection of survivin gene expression on frozen tissue sections obtained from breast cancer patients.

a. Expression of survivin gene was detected in different stages of breast cancer tissues.

Frozen tissue sections were fixed with acetone and incubated with survivin MB-Cy3. The sections were counterstained with Hoechst 33342 (blue nuclei) and then examined under a fluorescence microscope. As shown, survivin-expressing cells (red) were found in all stages of breast cancer tissues including DCIS, invasive carcinoma and lymph node metastases, but not found in normal breast tissues. Different sections from the same tissues were also stained

with a survivin antibody to confirm the presence of survivin positive cells (green). Yellow arrows indicate the detection of metastatic breast cancer cells in a lymph node by survivin MB or antibody.

b. Western blot analysis showed a high level of survivin protein (16.5 KDa) in primary breast cancer and lymph node with metastases but not in normal breast tissues.

c. Detection of survivin gene expression in tumor endothelial cells in breast cancer tissues using double-labeling survivin MB-Cy3 with an antibody to CD31.

Expression of survivin mRNA was labeled by survivin MB-Cy3 (Red) and tumor endothelial cells were labeled with an anti-CD31 antibody (Green). Another tissue section was double-labeled with goat anti-human survivin (FITC) and mouse anti-human CD31 antibodies (Red). A negative control section was stained with secondary antibody only without incubation with survivin-MB and primary antibody. All sections were counterstained with Hoechst 33342 (Blue). Arrows indicated that tumor endothelial cells expressed both survivin and CD31.

Figure 4. Detection of the levels of survivin gene expression in viable cells using survivin MB

a. Survivin MB-FITC produced green fluorescent signal in cytoplasma of breast cancer cells after transfection into viable cells. Treatment of the cancer cells with EGF for 1 hr or docetaxel for 24 hrs increased the fluorescence intensity in the cells. On the other hand, the fluorescence intensity generated by GAPDH MB-Cy3, which was cotransfected with the survivin MB, was relatively consistent in the cells.

b & c. The level of survivin or GAPDH mRNA in MB-transfected cells could be measured by FACScan analysis to determine the mean fluorescence unit for each sample. Relative level

of survivin mRNA was increased in EGF-stimulated cancer cell lines (green line), while there was no change in the fluorescence units detected in GAPDH MB transfected cells (red line). On the other hand, the level of survivin mRNA decreased in Adp53 vector - but not in AdCMV vector-transduced cells.

d. Real Time RT-PCR analysis showed that the level of survivin mRNA was increased by EGF treatment but decreased after over-expression of p53. The numbers in the figure represent the mean numbers from three repeat samples. The relative level of survivin gene expression was calculated as a ratio of the quantity of survivin and GAPDH PCR product.

Figure 5. Real time monitoring of the level of survivin gene expression in breast cancer cells.

Cells cultured in 96-well plates were transfected with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then added human EGF or Docetaxel. The fluorescence intensity in each well was measured at different time points following treatment using a fluorescence microplate reader. Each point in the curve is a ratio of the mean fluorescence unit of survivin MB (FITC, Ex/Em 480/530) and mean fluorescence unit of GAPDH MB (Cy3, Ex/Em 530/590) from four repeat samples. Similar results were obtained from three independent studies.

a. EGF treatment significantly increased the level of survivin mRNA (Student's t-test for all time points, $P<0.0005$). Analysis of the level of survivin protein by Western blotting further demonstrated that EGF increased survivin expression.

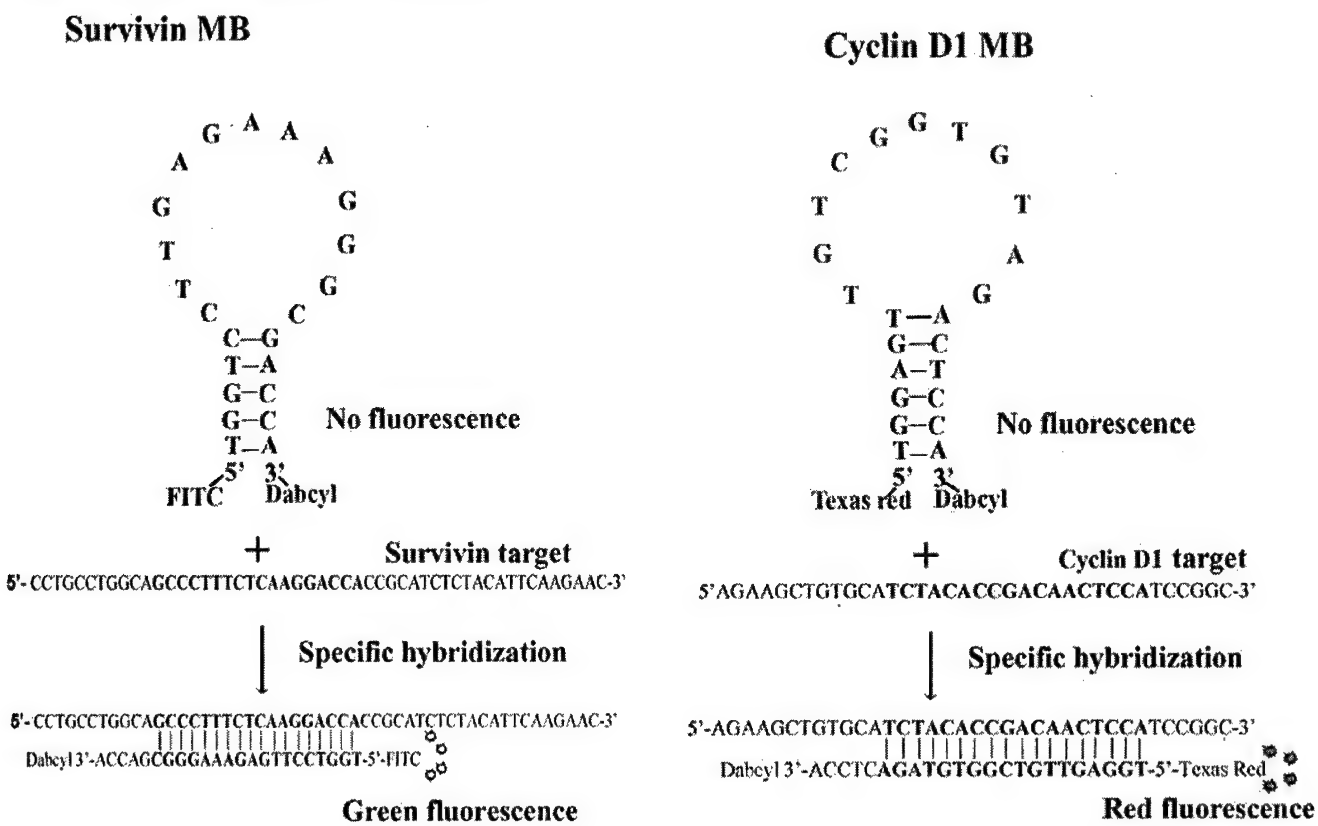
b. Docetaxel treatment increased the level of survivin gene expression in both tumor cell lines. A significant increase in the level of survivin mRNA was seen 24 to 48 hrs following the treatment (Student's t-test, $P< 0.05$). The levels of survivin gene expression and survivin

protein at different time points after treatment were also examined by Real Time RT-PCR (insert in the figure). Western blot analysis showed upregulation of survivin protein by docetaxel.

c. Transfection of survivin or control GAPDH MB into viable cells did not significantly alter the level of survivin protein as determined by Western blot analysis of cell lysates after transfected with either survivin MB or GAPDH MB for 24 hrs.

Figure 1. Schematic illustration of MB design and examination of specific binding of the MBs to their oligonucleotide targets.

a. Survivin and cyclin D1 MBs.



b. Examination of specificity of the MBs in vitro.

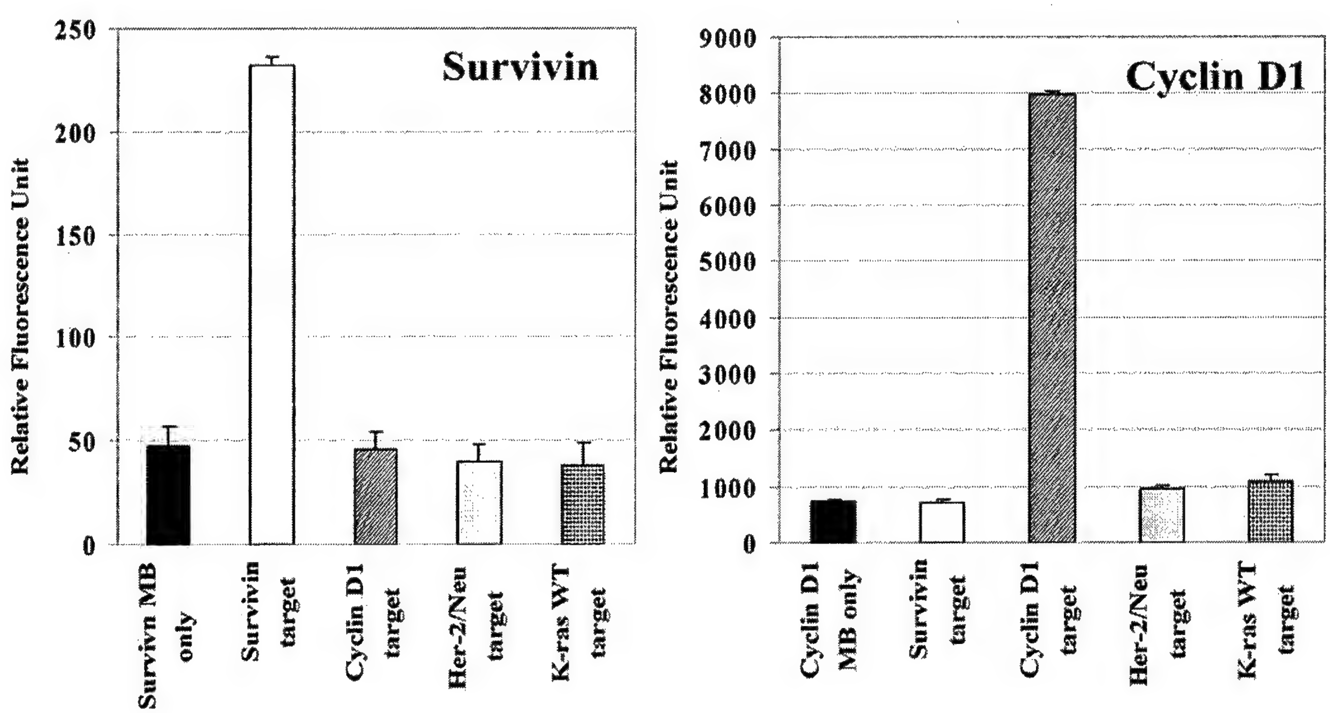


Figure 2. Simultaneous detection of the levels of survivin and cyclin D1 mRNAs in breast cancer cells.

a. Dual MB-imaging of breast cancer cells.

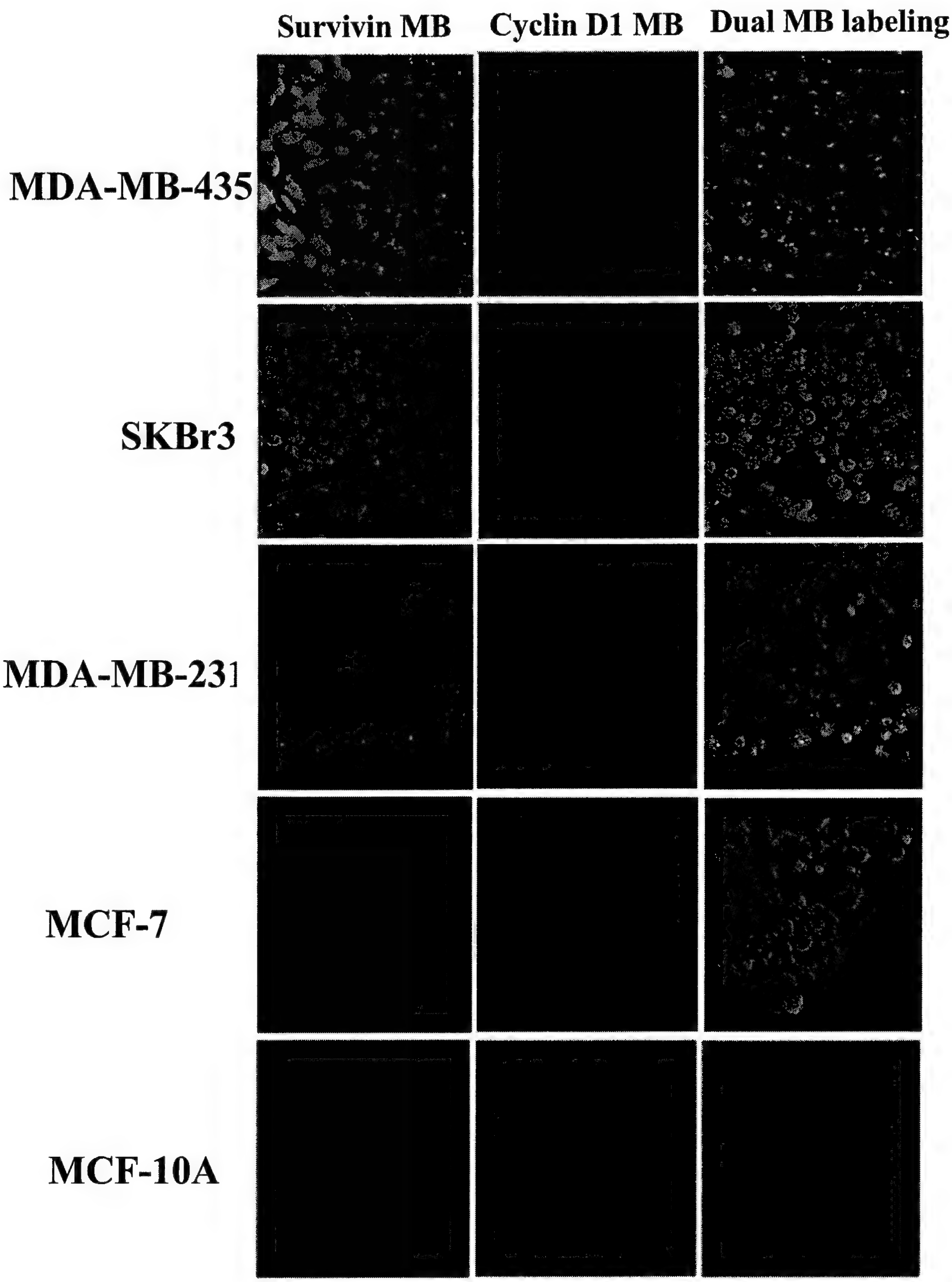
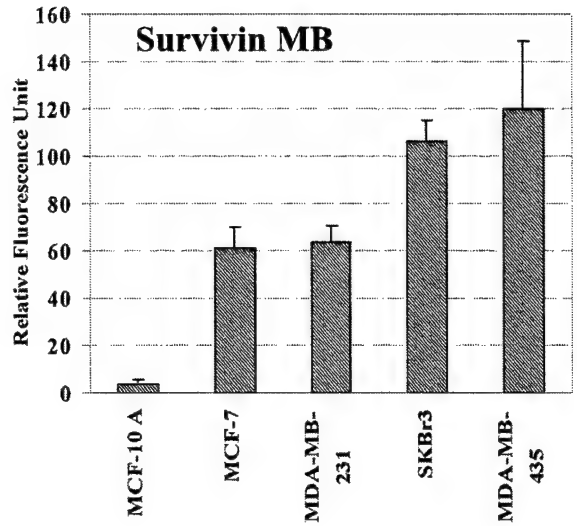
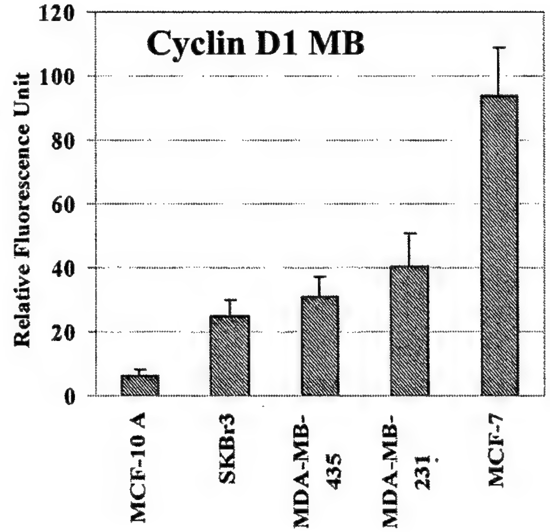


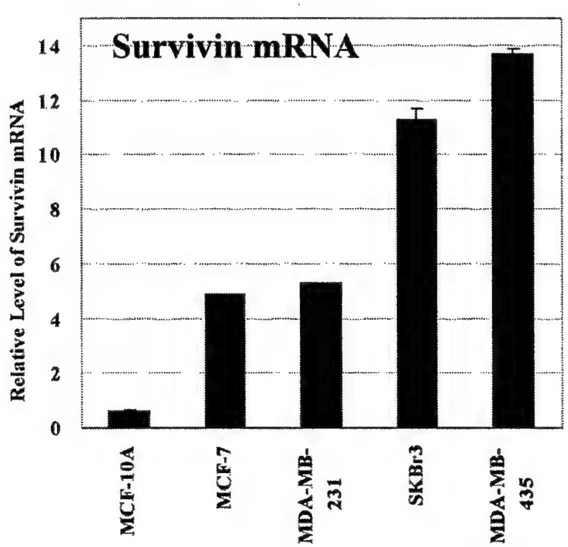
Figure 2

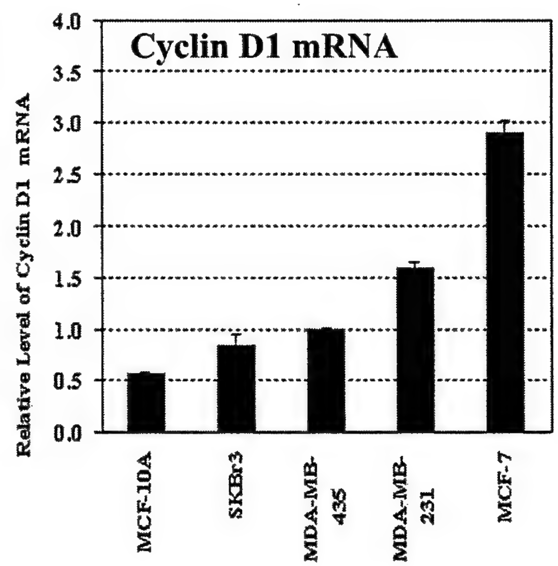
b. Quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells.





c. Detection of the levels of survivin and cyclin D1 mRNA by Real time RT-PCR.





d. Examination of the levels of survivin protein in tumor and normal cell lines by Western blotting.

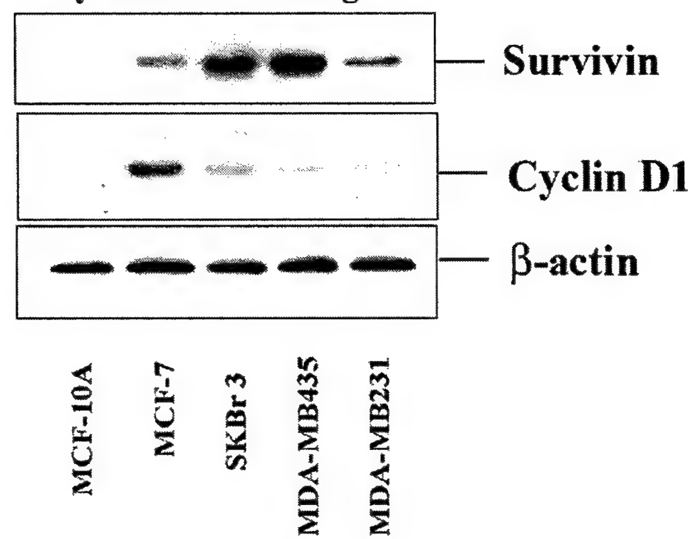
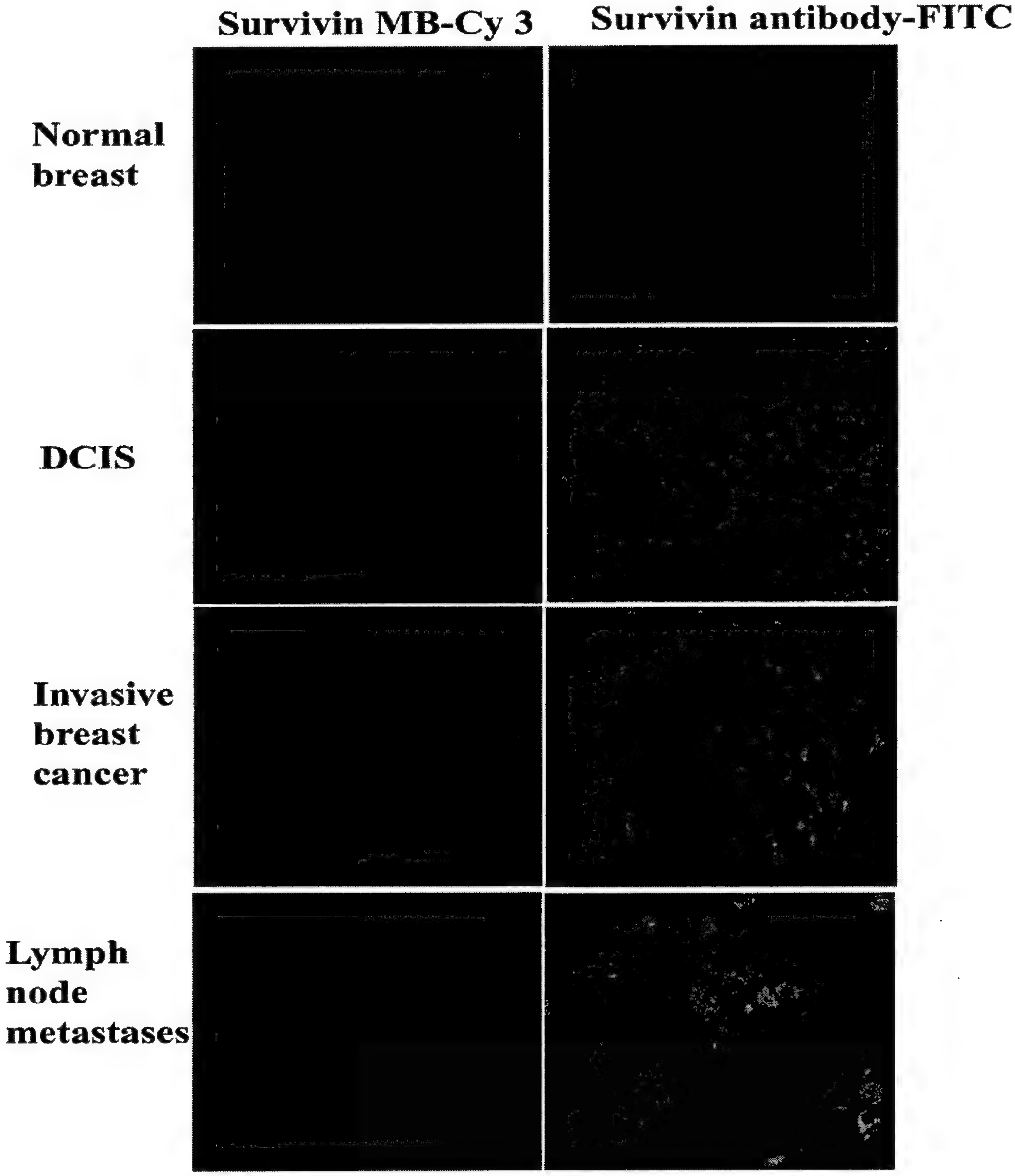


Figure 3. Detection of survivin gene expression on frozen tissue sections obtained from breast cancer patients.

a. Expression of survivin gene was detected in different stages of breast cancer tissues.



b. Western blot analysis showed a high level of survivin protein in breast cancer tissues.

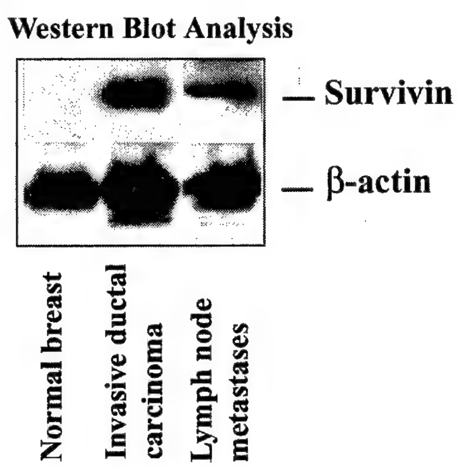


Figure 3

c. Detection of survivin gene expression in tumor endothelial cells in breast cancer tissues using double-labeling survivin MB-Cy3 with an antibody to CD31.

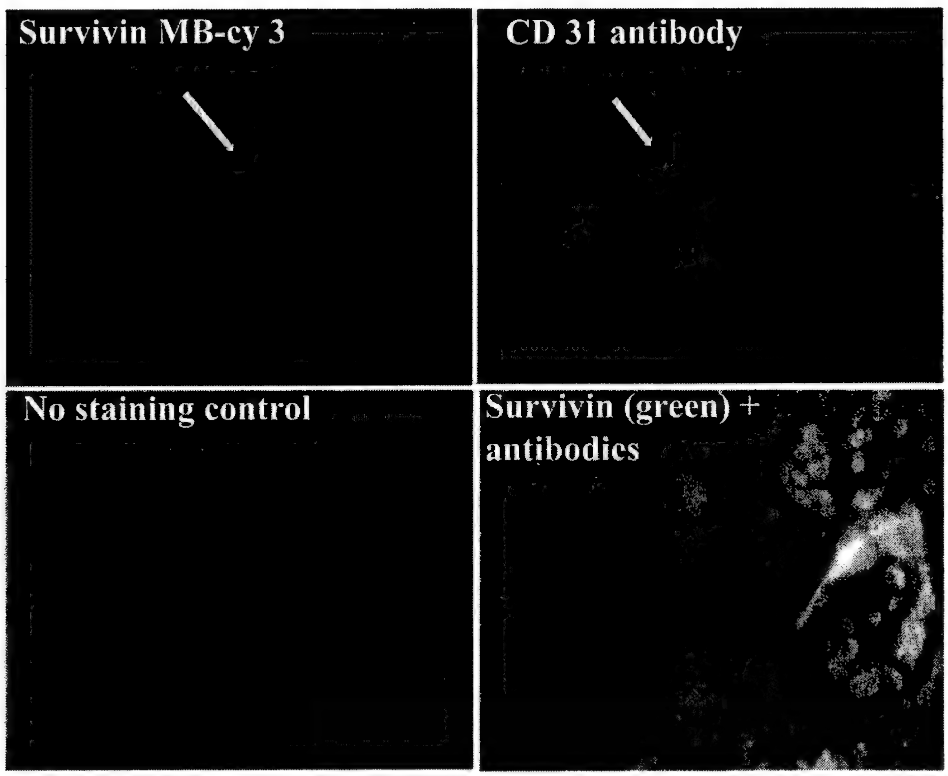


Figure 4. Detection of the levels of survivin gene expression in viable cells using survivin MB

a. Fluorescence imaging of the levels of survivin mRNA in breast cacner cells after EGF or docetaxel treatment

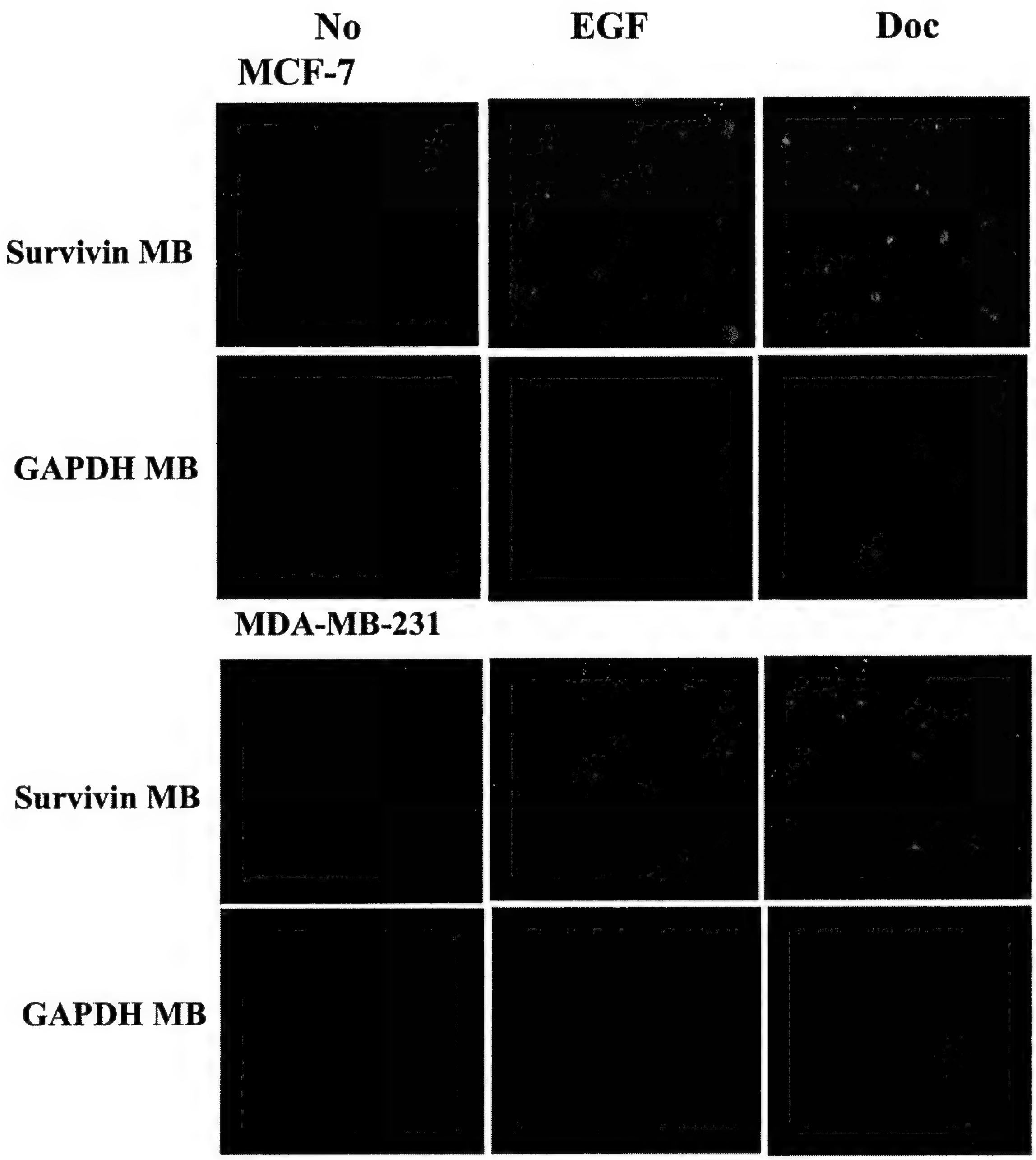
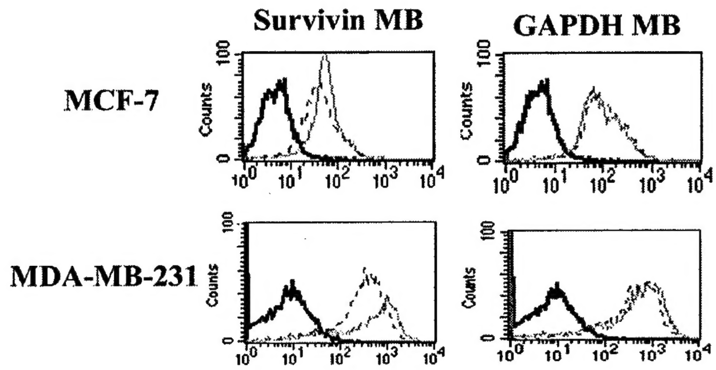


Figure 4 Detection of the levels of survivin gene expression in viable cells using survivin MB

b. Quantification of survivin mRNA in EGF-treated cells by FACScan analysis



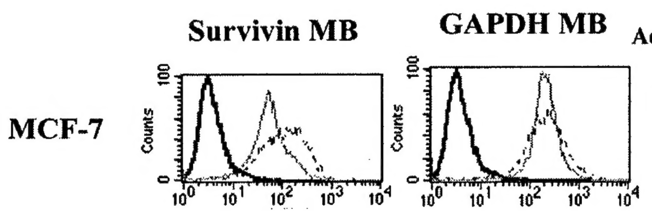
Relative level of survivin mRNA

(Survivin RFU/ GAPDH RFU)

	NO	EGF	Fold increase
MCF-7	0.35	0.49	1.4
MDA-MB-231	0.64	0.96	1.5

Blue: control, no MBs
Red: no EGF, survivin or GAPDH MBs
Green: 100 ng/ml of EGF, 1 hr, survivin or GAPDH MBs

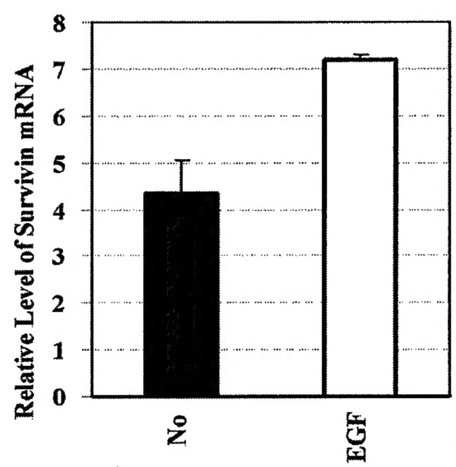
c. Detection of decreased survivin mRNA in Adp53-treansduced cells



	AdCMV	Adp53	Fold decrease
MCF-7	0.57	0.30	1.9

Blue: control, no MBs
Red: AdCMV control vector transduced, survivin or GAPDH MBs
Green: Adp53 vector transduced, survivin or GAPDH MBs

C. The levels of survivin mRNA determined by Real Time RT-PCR



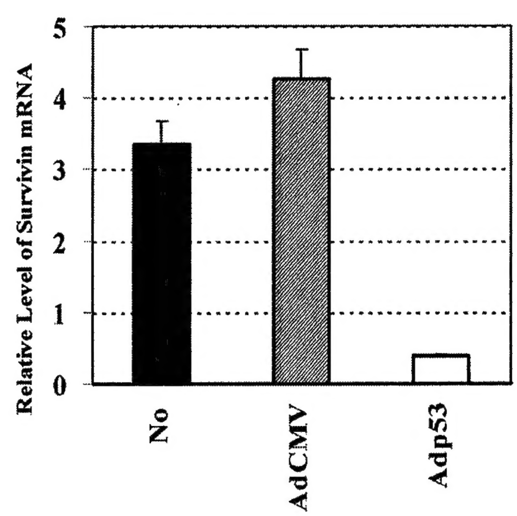
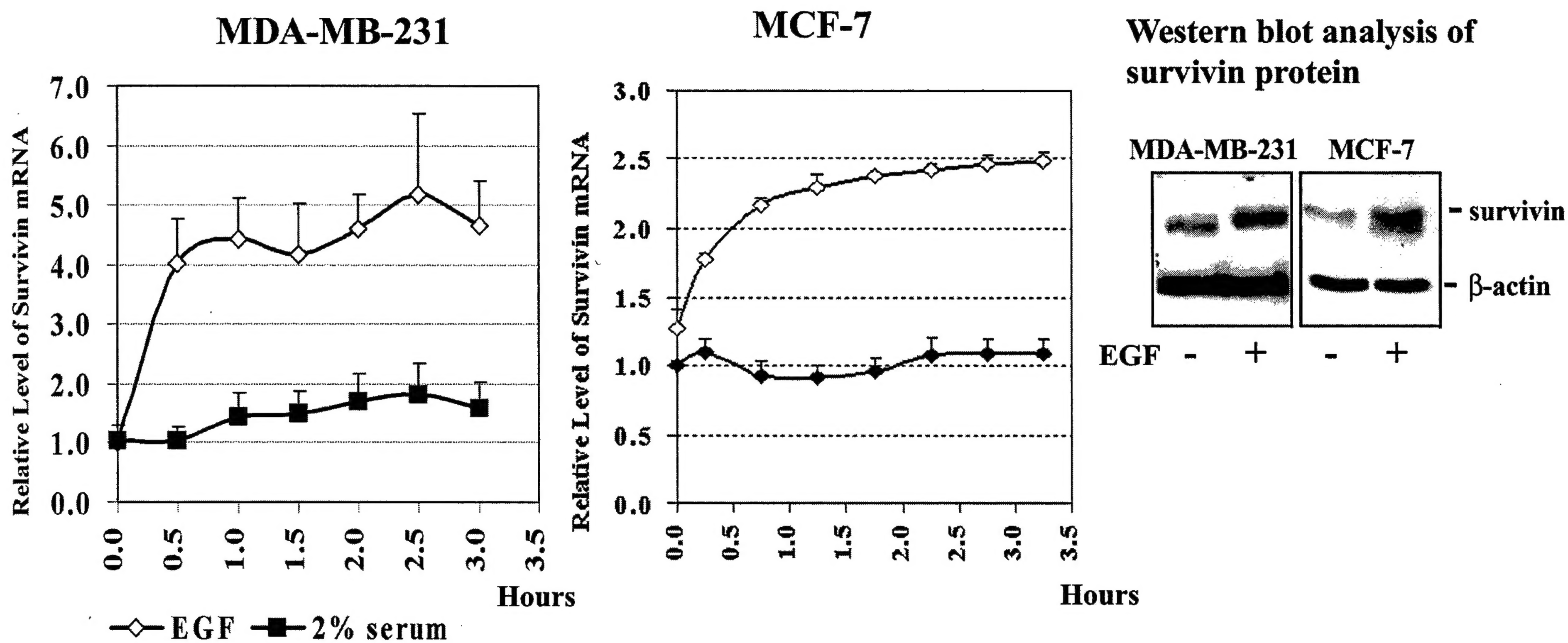


Figure 5. Real time monitoring of the level of survivin gene expression in breast cancer cells.
a. EGF treatment significantly increased the level of survivin mRNA.



Student's t-test: MDA-MB-231: $P = 0.0004$, MCF-7: $P = 2.5 \times 10^{-8}$

Figure 5 b. Docetaxel treatment increased the level of survivin gene expression in both tumor cell lines

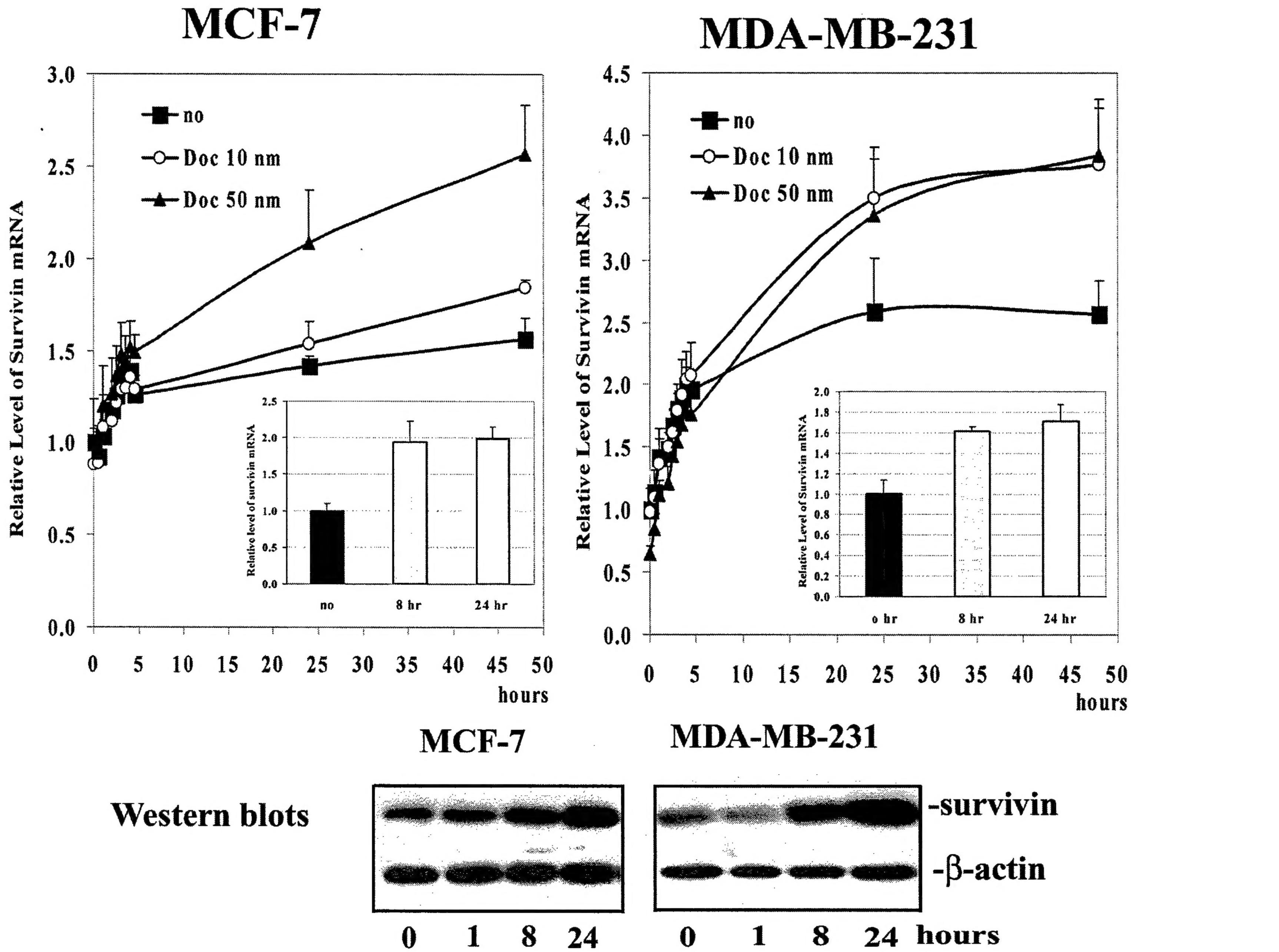


Figure 5 c

Transfection of survivin MB or a control GAPDH MB into viable cells did not significantly alter the level of survivin protein